Dose Response for TCDD Promotion of Hepatocarcinogenesis in Rats Initiated with DEN: Histologic, Biochemical, and Cell Proliferation Endpoints

R. R. Maronpot,[1] Julie F. Foley,[1] K. Takahashi,[1] T. Goldsworthy,[2] G. Clark,[1] A. Tritscher,[1] C. Portier,[1] and G. Lucier[1]

[1]National Institute of Environmental Health Sciences, Research Triangle Park, NC 27709 USA; [2]Chemical Industry Institute of Toxicology, Research Triangle Park, NC 27709 USA

The present study examines the dose–response relationship for 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD) promotion of histologic and biochemical parameters by using a two-stage model for hepatocarcinogenesis in female Sprague-Dawley rats initiated with a single intraperitoneal dose of 175 mg of diethylnitrosamine (DEN)/kg body weight at 70 days of age. Starting 2 weeks after initiation, treatment groups of 8–10 rats were given TCDD by gavage in corn oil once every 2 weeks for 30 weeks. Doses were 3.5, 10.7, 35.7, and 125 ng TCDD/kg body weight/day. A significant body weight reduction was present in the noninitiated group that received 125 ng TCDD. Relative liver weight was statistically increased in initiated rats treated with ≥ 10.7 ng TCDD and in noninitiated rats treated with ≥ 35.7 ng TCDD. Histopathologic evidence of cytotoxicity was dose-related in all TCDD-treated groups. There was a statistically significant dose response in the bromodeoxyuridine (BrdU) S-phase labeling index (LI) in the DEN-initiated rats ($p < 0.01$) and a marginally significant trend in the saline-treated rats ($p = 0.10$), but proliferating cell nuclear antigen S-phase LI and growth fraction within altered hepatic foci showed no increase. Among the DEN-initiated groups there was a significant increase in glutathione S-transferase altered hepatic foci stereological parameters in the 125 ng TCDD group. This study demonstrates that dose–response relationships for TCDD's effects on cell proliferation growth of altered hepatic foci are different from previously reported effects on P450 gene expression, indicating that different biological or biochemical responses may exhibit different dose–response relationships. This implies that the shape of the dose–response curve for receptor-mediated carcinogens, such as TCDD, might not be predicted solely on the basis that a response is receptor mediated. *Key words:* cell proliferation, clinical chemistry, focus stereology, initiation, labeling index, liver foci, promotion. *Environ Health Perspect* 101:634–642(1993)

Two-stage hepatocarcinogenesis models in rats are useful in identifying tumor-promoting activity of chemicals by stereologic quantification of altered hepatocellular foci (*1,2*). In addition, chemically induced cell proliferation may play a critical role in the development of cancer, especially for chemicals that appear to act by nongenotoxic mechanisms (*3–6*). 2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD), a hepatocarcinogen in female rats (*7,8*), is generally regarded as a nongenotoxic carcinogen (*9–11*) and has been identified as a potent tumor promoter in two-stage rat liver tumor models with negligible tumor-initiating potential (*12–14*). Most risk assessments for estimating human health risks from TCDD exposure are derived from tumor incidence data in female rat liver (*15*).

Although mechanisms for the carcinogenic activity of TCDD and its structural analogs are unknown, many toxic and biochemical effects of TCDD appear to require the Ah receptor, including effects on signal transduction pathways possibly involving epidermal growth factor receptor (*16,17*) and estrogen receptor (*18,19*). Induction of liver tumors in female rats treated with TCDD is associated with enhanced hepatocyte proliferation and is modulated by ovarian hormones (*20*). Because cell proliferation may play a role in the carcinogenic process and because evaluation of altered hepatic foci (AHF) is useful in identifying hepatocarcinogens, information on dose–response relationships for these effects within the framework of a two-stage model for hepatocarcinogenesis may provide essential information for estimating cancer risks from dioxin exposure.

Unfortunately, there is no information in the literature on dose–response relationships for TCDD's effects on cell proliferation either in hepatocytes or within AHF, although stereological measures of enzyme-altered foci have been reported (*12*). However, there is considerable data on dose–response relationships for induction of two cytochrome P450 isozymes (CYP1A1 and 1A2) in rat liver (*9,21–23*). Although the mechanistic link between P450 induction and cell proliferation or cancer is not readily apparent, we have compared dose–response relationships for these different endpoints to evaluate relative sensitivities for various responses to TCDD within the framework of a tumor promotion model.

In the present studies, we used a two-stage model for hepatocarcinogenesis in female Sprague-Dawley rats to determine the dose response of TCDD promotion, as measured by histologic and biochemical parameters, after initiation with a single dose of diethylnitrosamine (DEN). After 30 weeks of TCDD treatment, several parameters were assessed including histopathology, AHF positive for the placental isozyme of glutathione S-transferase (PGST), hepatocyte proliferation, and liver TCDD concentrations. We evaluated dose–response relationships on the basis of both administered dose and target tissue dose of total (free plus bound) TCDD. Furthermore, dose–response relationships for $PGST^+$ foci and hepatocyte proliferation were compared to those for CYP1A1 and CYP1A2 induction in the same livers to determine if enzyme induction is correlated with effects on cell proliferation and size and number of $PGST^+$ foci. In addition, we compared cell proliferation rates in focal lesions to those in normal hepatocytes to assess TCDD's effects on selective growth enhancement of putative precursors of neoplastic lesions. These studies revealed that there was considerable interindividual variation in TCDD's effects on hepatocyte proliferation, various parameters of $PGST^+$ foci, and histomorphologic alterations. It appears that these biological responses occur at higher TCDD exposures than previously reported for CYP1A1 and CYP1A2 induction in rat liver (*22*).

Materials and Methods

Female Sprague-Dawley rats, used because of a previous carcinogenicity bioassay (*7*) and liver tumor promotion studies (*12,24*), were obtained from Charles River Breeding Laboratories, Inc. (Raleigh, North Carolina), acclimatized for 2 weeks, and randomly distributed into 1 of 10 treatment groups on the basis of body weight. The treatment groups consisted of four separate doses of TCDD after initiation with DEN and appropriate noninitiated and vehicle controls as detailed in Table 1. At 70 days of age, 175 mg DEN/kg body weight was administered intraperitoneally as the initiating agent with 1 µl saline/g body weight as the DEN vehicle control. Starting 2 weeks after initiation, rats were given TCDD by gavage in corn oil once every 2 weeks for 30 weeks. Biweekly doses ranged from 49 to 1750 ng TCDD/kg and are considered equivalent to 3.5 to 125 ng TCDD/kg body weight/day. Rats were killed 7 days after the final treatment. There were 8–10 rats/group. The high dose was previously shown to promote liver tumors in DEN-initiated rats (*24*).

Address correspondence to R. R. Maronpot, NIEHS, PO Box 12233, Research Triangle Park, NC 27709 USA.

Received 14 June 1993; accepted 17 September 1993.

Table 1. Final body weight (BW), liver weight (LW), and hepatocyte labeling index (LI) in female Sprague-Dawley rats

Treatment group	*n*	Body weight (g)[a]	Liver weight (g)[a]	LW/BW (× 100)[b]	LI(%)[c]
DEN/corn oil	10	391 ± 45	12.7 ± 1.4[d]	3.3 ± 0.3[d]	5.28 ± 2.22[d]
DEN/TCDD (3.5 ng/kg)	9	387 ± 43	13.3 ± 2.6	3.4 ± 0.4	3.28 ± 1.55*
DEN/TCDD (10.7 ng/kg)	9	390 ± 71	14.1 ± 2.4	3.6 ± 0.3*	3.25 ± 2.91
DEN/TCDD (35.7 ng/kg)	8	355 ± 37	13.4 ± 1.1	3.8 ± 0.3*	6.39 ± 3.62
DEN/TCDD (125 ng/kg)	9	362 ± 68	16.0 ± 2.4*	4.5 ± 0.3*	14.35 ± 8.26*
Saline/corn oil	9	442 ± 47[d]	14.9 ± 2.1	3.4 ± 0.2[d]	3.41 ± 1.97
Saline/TCDD (3.5 ng/kg)	9	399 ± 38	13.9 ± 1.7	3.5 ± 0.2	3.22 ± 2.16
Saline/TCDD (10.7 ng/kg)	9	402 ± 67	14.3 ± 2.0	3.6 ± 0.3*	4.87 ± 5.66
Saline/TCDD (35.7 ng/kg)	9	433 ± 88	16.5 ± 3.9	3.8 ± 0.5*	5.33 ± 5.82
Saline/TCDD (125 ng/kg)	9	346 ± 34*	15.1 ± 4.7	4.3 ± 1.2*	7.09 ± 8.15

DEN, diethylnitrosamine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[a]TCDD effect and initiation effect significant ($p < 0.05$) in analysis of variance.
[b]TCDD effect significant ($p < 0.05$) in analysis of variance.
[c]TCDD effect and interaction between TCDD and initiation significant ($p < 0.05$) in analysis of variance.
[d]$p < 0.05$ for trend test using a general linear model.
*Statistically significant versus control as measured by two-tailed, unpaired Student's-*t* test, $p < 0.05$.

We weighed the liver at necropsy and froze representative samples of the left and right median, left lateral, and right posterior lobes in liquid nitrogen or fixed them in 10% formalin for routine hematoxylin and eosin staining and histopathologic evaluation using published diagnostic criteria (*25*). A section of duodenum was also saved and embedded along with the fixed liver sections as a positive control for the cell proliferation studies. Histologic sections of liver were stained for PGST using an immunohistochemical procedure (*26*). A minimum of eight contiguous $PGST^+$ hepatocytes was required for a $PGST^+$ focus to be scored. Foci were quantified using computer-assisted image analysis (NIH Image V3.8 for the Macintosh by W. Rasband, NIH) and subjected to stereological analysis as described previously (*27,28*). We estimated the number and size of AHF in H&E-stained sections and the relative degree of toxicity based on a semiquantitative grading scale (see Table 3 for details).

Immediately before euthanizing the rats, blood was collected under carbon dioxide anesthesia by cardiac puncture using a 20-gauge needle and a 10-ml syringe. Blood was transferred to prelabeled serum separator tubes without anticoagulant, and sera were harvested from the clotted blood within 30 min of collection. The following clinical chemistry measurements were made using a Monarch 2000 (Instrumentation Laboratories, Inc., Lexington, Massachusetts) and commercially available reagents: alkaline phosphatase (AP), glucose, alanine aminotransferase (ALT), total cholesterol, triglycerides, sorbitol dehydrogenase (SDH), 5′-nucleotidase (5′-Nuc), and total bile acids. Sera were stored at -70°C between collection and measurements.

Replicative DNA synthesis (S-phase hepatocytes) was immunohistochemically quantified by identifying bromodeoxyuridine (BrdU) incorporation after subcutaneous implantation of a 7-day osmotic minipump (Alzet model 2ML1; 10 µl/hr; Alzet Corp., Palo Alto, California) filled with 30 mg/ml BrdU in distilled water. We surgically implanted minipumps 7 days before sacrifice while rats were under methoxyfluorane/oxygen inhalation anesthesia. Incorporation of BrdU was quantified in immunohistochemically stained sections (*29*) by counting labeled nuclei among at least 1000 hepatocytes in randomly selected fields. For determination of hepatic cell proliferation indices among various treatment groups, care was taken to avoid AHF in the fields counted. A labeling index was generated by dividing the number of labeled hepatocytes by the total number of hepatocytes counted, and the result was expressed as a percentage. Before counting, written criteria for scoring were used by four independent observers to determining the labeling index for three rats. There was close agreement in the independently determined labeling indices. The lobular distribution of S-phase hepatocytes was noted in most cases, although this was not done for some livers with only a small number of labeled cells.

Labeling indices determined from slides stained immunohistochemically for proliferating cell nuclear antigen (PCNA) (*30,31*) and counterstained with H&E were evaluated in 10–12 AHF from rats given DEN followed by corn oil or one of the TCDD treatment regimens. Positive staining was evaluated in 90–100% of the hepatocytes in each focal transection according to previously published criteria (*30,31*). All foci evaluated were clearly discernible and were randomly selected as representative medium-sized, clear-cell AHF (0.3–1.25 mm in diameter) as measured using a calibrated eyepiece reticule. We calculated a PCNA S-phase labeling index plus a growth fraction (the proportion of cells in G_1, S-phase, G_2, and mitosis combined) for each AHF evaluated. We analyzed 1-g samples of liver for TCDD concentrations by GC-MS as previously reported (*20*).

Results

Body and Liver Weight

At the end of the study, body weight gain was statistically significantly lower than controls in the noninitiated group that received 125 ng TCDD/kg body weight/day. There was a nonsignificant treatment-related decreased trend in body weight in initiated rats ($p = 0.18$) and a significant decrease in body weight in the noninitiated rats ($p = 0.004$). Data are presented in Table 1 and illustrated in Figure 1. A continuous mathematical model was fit to these data to give an indication of TCDD-induced changes in overall body weight. A model which fit the data well is:

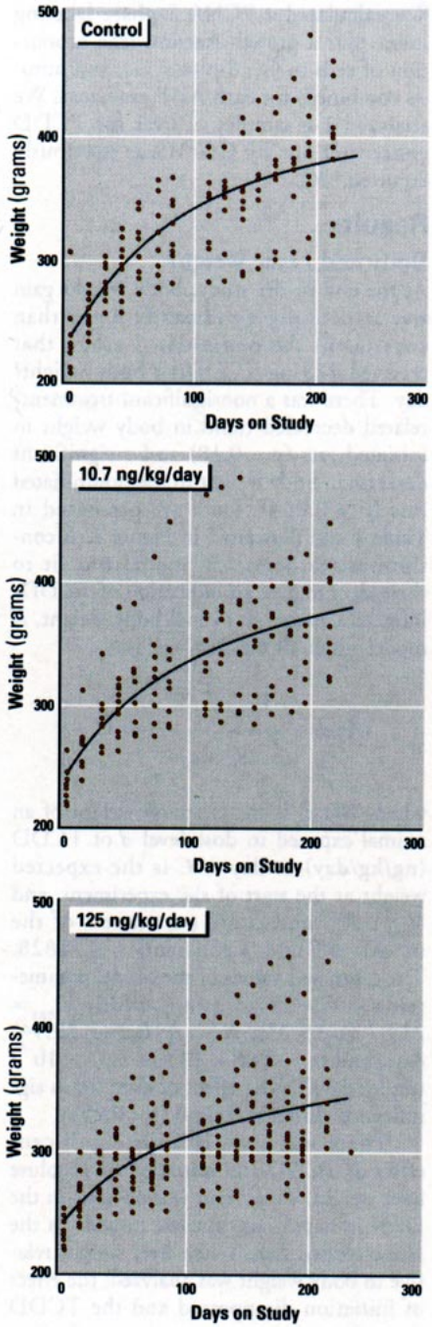
$$W(t,d) = \frac{V_{max}\, tE^{(\alpha d)}}{K_m + t} + C$$

where $W(t,d)$ is the expected weight of an animal exposed to dose level d of TCDD (ng/kg/day) at day t, C is the expected weight at the start of the experiment, and V_{max}, K_m, and α are parameters of the model. E (Euler's constant) = 2.71828. The estimated values of the model parameters are C = 237.3 g (± 4.4 SE), V_{max} = 219.7 (± 15.73), K_m = 116.3 (± 22.94) days, and $\alpha = -2.86 \times 10^{-3}$ ($\pm 3.57 \times 10^{-4}$) $(ng/kg/day)^{-1}$. The effect of dose (α) is significantly different from 0 ($p < 0.05$).

There was a statistically significant effect of TCDD and initiation on absolute liver weight. This trend was obvious in the DEN initiated rats, but less notable in the saline-treated rats. When liver weight relative to body weight was analyzed, the effect of initiation disappeared and the TCDD dose response became apparent and effectively equal in the initiated and noninitiated groups. Relative liver weight was statistically increased over controls in rats treated with ≥ 10.7 ng TCDD/kg body weight/day in the initiated animals and in rats treated with ≥ 35.7 ng TCDD/kg body weight/day in the noninitiated animals. The ratio of liver weight to body weight can also be modeled using a simple form given by:

$$R(d) = \frac{V_{max}\, d}{K_m + d} + C$$

where $R(d)$ is (liver weight/body weight) × 100% at dose d (ng/kg/day), C is the ratio in control animals and V_{max} and K_m are



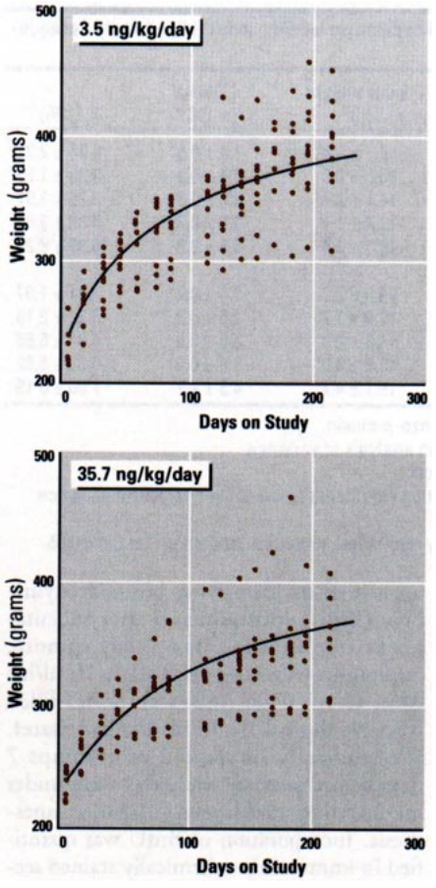


Figure 1. (above and left) The effects of TCDD on body weight as a function of age and level of exposure.

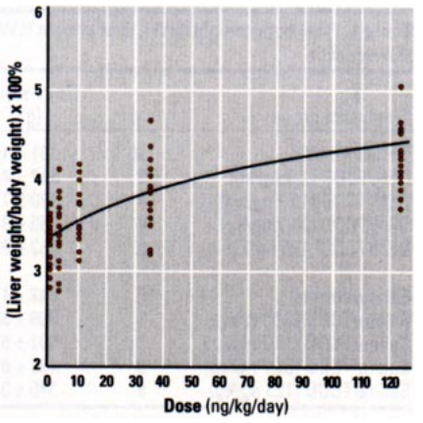


Figure 2. Effect of TCDD on the ratio of the liver weight to the body weight.

parameters. For the data given here, estimates are C = 3.361 (± 0.0815)%, V_{max} = 1.792 (± 0.692)%, and K_m = 92.31 (± 80.16) ng/kg/day. The fit of this model to the data is illustrated in Figure 2. The value of V_{max} is significantly different from zero ($p < 0.05$), indicating the significant effect of treatment on this ratio. The maximum change indicated by the model is 1.792% for a large treatment dose. At 125 ng TCDD/kg body weight/day, the change over control is 1.03%.

Gross Lesions

Livers from rats treated with either 35.7 or 125 ng TCDD/kg body weight/day were either uniformly darker than normal or had 2–5 mm irregularly round, dark areas on all natural surfaces. A 3-mm tan focus, diagnosed as a hepatocellular adenoma, was observed on a cut surface of the left median lobe of a rat in the DEN/TCDD (125 ng/kg) group, and a 10-mm tan nodule, diagnosed as focal fibrosis, was present on the left median lobe of a rat in the DEN/TCDD (35.7 ng/kg) group. Biliary cysts were present in the livers of two rats, one from the DEN/TCDD (10.7 ng/kg) group and the other from the DEN/TCDD (125 ng/kg) group. Livers from all other rats were macroscopically normal. The frequency of hepatocellular proliferative lesions is not significantly different from that which might be expected in controls at a 30-week time point. Biliary cysts commonly occur in TCDD-treated rats (unpublished observations). Based upon previous studies, liver tumor incidence was increased after a total of 60 weeks of treatment with TCDD (125 ng/kg/day) in DEN-initiated rats (*24*).

Clinical Pathology

Means, standard deviations, and the analysis of the serum chemistry data are presented in Table 2. Statistically significant dose–response changes due to TCDD were seen for AP, total cholesterol, triglycerides, SDH, and 5′-Nuc. In addition, significant effects of initiation were seen for AP and triglycerides and a marginally significant effect on SDH (p = 0.058) was seen. There were no statistically significant interactions between DEN and TCDD for any of the serum chemistry measurements, although many of the measures showed similar high dose responses but significantly different control responses between initiated and noninitiated rats. ALT was marginally affected by TCDD (p = 0.087) with a slight interaction between TCDD and DEN (p = 0.073) and with statistically increased response over control in the two highest dose groups of initiated rats. Glucose and total bile acids were unaffected by either TCDD or DEN treatments. The elevations of ALT, AP, 5′-Nuc, and SDH are consistent with the cytotoxicity observed in histologic sections.

Histopathology

Histologic evidence of toxicity consisted of cytoplasmic vacuolization, fatty change, bile duct hyperplasia, and pigment in Kupffer cells and is documented for individual rats in Table 3. There was a dose-related increase in toxicity in both initiated and noninitiated rats, with somewhat greater severity of toxicity in noninitiated rats. An estimate of AHF as detected in H&E-stained sections based on number and size is also presented in Table 3 for individual rats. Rats initiated with DEN had more AHF in H&E-stained sections than noninitiated rats. Most AHF were clear cell, acidophilic, or basophilic. There was no strong positive or negative correlation between individual rat BrdU S-phase hepatocyte labeling index (LI) and H&E-stained AHF (r = 0.321 for initiated and r = 0.433 for noninitiated rats) or between labeling index and toxicity (r = 0.533 for initiated and r = 0.451 for noninitiated rats). Likewise, there was no clear correla-

Table 2. Serum clinical chemistry parameters from female Sprague-Dawley rats

Group	AP (IU/l)[a]	Glucose (mg/dl)	ALT (IU/l)	Total cholestrol (mg/dl)[b]	Triglycerides (mg/dl)[a]	SDH (IU/l)[b]	5′-Nuc (IU/l)[a]	TBA (μmol/l)
DEN/corn oil	75.5 ± 25.9[c]	156.0 ± 15.4	23.3 ± 5.6[c]	102.6 ± 21.5[c]	303.2 ± 129.7[c]	17.1 ± 8.8[c]	30.5 ± 6.0[c]	40.98 ± 26.80
DEN/TCDD (3.5 ng/kg)	75.2 ± 26.1	150.7 ± 10.2	28.9 ± 14.1	106.7 ± 22.0	374.9 ± 146.7	21.4 ± 10.5	31.7 ± 10.7	45.01 ± 29.57
DEN/TCDD (10.7ng/kg)	76.6 ± 21.8	154.7 ± 17.8	34.9 ± 25.5	118.7 ± 12.2	298.1 ± 92.9	22.9 ± 22.0	39.9 ± 17.9	29.71 ± 13.20
DEN/TCDD (35.7 ng/kg)	98.0 ± 25.8	149.0 ± 25.6	35.5 ± 12.1	134.4 ± 12.3	284.3 ± 79.7	29.6 ± 12.0	47.0 ± 26.6	41.34 ± 8.58
DEN/TCDD (125 ng/kg)	151.7 ± 49.1	145.1 ± 14.2	41.4 ± 14.8	141.6 ± 25.9	208.0 ± 82.7	38.2 ± 11.0	48.8 ± 10.3	52.89 ± 28.14
Saline/corn oil	99.0 ± 46.3[c]	144.0 ± 8.6	37.7 ± 14.3	106.2 ± 17.2[c]	432.7 ± 105.9	29.6 ± 15.4	35.2 ± 6.2[c]	32.30 ± 13.15
Saline/TCDD (3.5 ng/kg)	104.0 ± 55.9	160.2 ± 18.1	28.9 ± 9.6	96.3 ± 10.8	354.9 ± 144.8	25.8 ± 14.9	32.9 ± 11.5	43.77 ± 24.34
Saline/TCDD (10.7 ng/kg)	99.3 ± 68.3	165.4 ± 22.0	34.9 ± 8.4	111.4 ± 29.9	403.1 ± 191.8	24.1 ± 8.8	32.7 ± 6.6	42.84 ± 25.51
Saline/TCDD (35.7 ng/kg)	117.0 ± 84.8	156.3 ± 13.2	35.2 ± 8.6	132.9 ± 33.9	486.3 ± 379.8	31.1 ± 12.3	51.0 ± 20.5	30.11 ± 7.04
Saline/TCDD (125 ng/kg)	151.4 ± 55.2	154.7 ± 17.6	33.6 ± 7.0	144.8 ± 27.2	316.6 ± 96.5	33.4 ± 9.6	56.1 ± 21.1	42.11 ± 19.25

AP, alkaline phosphatase; ALT, alanine aminotransferase; SDH, sorbitol dehydrogenase; 5′-Nuc, 5′-nucleotidase; TBA, total bile acids; DEN, diethylnitrosamine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[a]TCDD effect and initiation effect significant ($p < 0.05$) in analysis of variance.
[b]TCDD effect significant ($p < 0.05$) in analysis of variance.
[c]$p < 0.05$ for trend test using a general linear model.
*Statistically significant versus control as measured by two-tailed, unpaired Student's-*t* test, $p < 0.05$.

tion between the presence of H&E-stained AHF and toxicity ($r = 0.206$ for initiated and $r = 0.213$ for noninitiated rats). The highest mean BrdU LIs for non-AHF hepatocytes were present in the groups receiving the highest dose of TCDD, even though LI was poorly correlated with AHF or toxicity on an individual rat basis.

Cell Proliferation

Mean BrdU hepatocyte LIs for the different treatment groups are summarized in Table 1 and individual rat LIs are given in Table 3. For all rats initiated with DEN, $BrdU^+$ S-phase nuclei were randomly distributed throughout the hepatic lobules. In contrast, there was a periportal distribution of $BrdU^+$ S-phase nuclei in several noninitiated TCDD-treated rats (Table 3). Overall, there was a statistically significant increased trend in LI as a function of dose of TCDD with an interaction between TCDD and DEN. This trend suggests dose dependency, but the results when compared to the controls are not statistically significant. The trend in increasing LI was stronger in initiated rats than in noninitiated rats. There was a statistically significant decrease in LI in the low-dose group of initiated rats. This may be a reflection of the increased LI in DEN-treated control rats; an increase which is marginally significant ($p = 0.07$) compared to the noninitiated controls.

The variability in LIs between rats within a given dose group increased as the dose of TCDD was increased and was especially evident in the 125 ng/kg dose group, where there were rats with a clearly elevated LI and other rats which were not different from controls (Fig. 3). This variability was also evident when individual rat LIs were plotted against the liver concentration of TCDD (Fig. 4).

PCNA + S-phase hepatocyte LIs were enumerated within AHF evident in H&E-stained sections from DEN-initiated rats, and the results for individual AHF are presented along with the growth fraction and the AHF diameter in Table 4. It was apparent that most AHF generally had a higher S-phase LI and growth fraction than the surrounding nonfocal hepatocytes (data not presented). There was no clear correlation between AHF diameter and PCNA + S-phase LI ($r = 0.157$) or growth fraction ($r = 0.217$), whereas the correlation between PCNA + S-phase and growth fraction was $r = 0.822$). No significant treatment-related effect on either S-phase ($p = 0.520$) or growth fraction ($p = 0.676$) LI was found for AHF based on analysis of variance; thus, TCDD treatment did not increase the LI within AHF selected for evaluation.

$PGST^+$ Foci of Cellular Alteration

Stereologic results obtained from quantification of AHF positively stained for PGST are summarized in Table 5. All five measures in Table 5 showed a highly significant effect ($p < 0.01$) of initiation when analyzed in the analysis of variance. The number of foci/cm^2, volume fraction, and mean focal volume also showed significant TCDD dose–response trends ($p < 0.05$) and significant interaction between TCDD and DEN ($p < 0.05$). For all calculated parameters, rats initiated with DEN had higher values than comparable noninitiated groups. This expresses itself as a significant initiation effect for all five measures. Among the DEN-initiated groups there was a significant dose-related increase in foci/cm^2, volume fraction, and mean focus volume. The absence of a significant increase in foci/cm^3 is a consequence of the 125 ng TCDD group having larger foci, as reflected by the mean focus volume. There were no individual significant dose–related increases in $PGST^+$ focus stereological parameters in the noninitiated rat groups. This lack of significance is due to a reduction in focal lesion production in the high exposure noninitiated group.

Although the $PGST^+$ focus response shows a clear treatment-related response, when individual rat stereological calculations such as focus size are plotted against administered dose of TCDD, there is obvious variability in this response with one or two rats in each TCDD-treated group being especially responsive (Fig. 5). The correlation between focus size and liver concentration of TCDD is somewhat better (Fig. 6) but still demonstrates individual rat variability. Other stereological parameters such as numbers of foci and volume fraction show similar responses and variability to that demonstrated for focus size in Figures 5 and 6.

Hepatic TCDD Concentrations

TCDD concentrations in liver were quantified only in DEN/TCDD-treated rats because previous studies had demonstrated that TCDD concentrations were similar in DEN-initiated and in noninitiated rats (*20*). In general, within the dose range used (3.5–125 ng TCDD/kg/day) there was a linear relationship between administered dose and liver concentration whether TCDD levels were based on liver wet weight or liver lipid (Fig. 7) (*22*). Average values on a wet-weight basis were approximately 0.5 ppb in the 3.5 ng/kg dose group and approximately 20 ppb in the 125 ng/kg dose group.

Discussion

Two-stage hepatocarcinogenesis models in rats may be used to test the tumor promotion ability of a chemical (*1*). The first morphologic indication of a chemically induced carcinogenic process in rat liver is the appearance of putatively preneoplastic

Table 3. Bromodeoxyuridine S-phase labeling indices (BrdU LI), altered hepatic foci (AHF), and toxicity

Group	Animal ID	BrdU LI (%)[a]	AHF[b]	Toxicity[c]	Group	Animal ID	BrdU LI (%)[a]	AHF[b]	Toxicity[c]
DEN/corn oil	1-1	7.69r	2	0	Saline/corn oil	19-1	5.71c	0	0
	1-2	3.70r	3	0		19-2	4.59r	0	0
	1-3	2.30r	1	0		19-3	5.36r	1	0
	1-4	3.31r	2	0		20-1	0.97	0	0
	2-1	5.41r	1	0		20-2	2.45r	0	0
	2-2	4.32r	1	0		20-3	3.22r	0	0
	2-3	3.13r	1	0		21-1	5.61p	0	0
	3-1	8.48r	2	0		21-2	1.77	0	0
	3-2	7.03r	2	0		21-3	0.99	0	0
	3-3	7.38r	3	0					
DEN/3.5 ng TCDD/kg/day	4-1	3.34r	2	0	Saline/3.5 ng TCDD/kg/day	22-1	2.81m	0	0
	4-2	5.61r	2	0		22-2	0.62	0	0
	4-3	5.65r	1	0		22-3	1.75	0	0
	5-1	4.20r	3	1		23-1	0.98	0	0
	5-2	2.38r	3	2		23-2	1.81r	0	0
	5-3	2.66r	3	0		23-3	6.42r	0	0
	6-1	1.77	2	0		24-1	3.59r	1	0
	6-2	2.32r	3	1		24-2	5.87r	1	2
	6-3	1.59	2	0		24-3	5.16p	0	0
DEN/10.7 ng TCDD/kg/day	7-1	1.96	1	0	Saline/10.7 ng TCDD/kg/day	25-1	19.29c	0	0
	7-2	0.2	0	2		25-2	2.57p	0	0
	7-3	10.10r	3	0		25-3	5.02p	0	0
	8-1	3.48r	0	1		26-1	1.86p	0	1
	8-2	2.89r	1	0		26-2	1.43p	0	1
	8-3	2.91r	3	1		26-3	4.41p	0	0
	9-1	1.90r	1	0		27-1	5.76m	0	2
	9-2	2.30r	3	0		27-2	0.85	0	0
	9-3	0.49	2	0		27-3	2.60p	0	2
DEN/35.7 ng TCDD/kg/day	10-1	5.11r	2	0	Saline/35.7 ng TCDD/kg/day	28-1	9.07r	0	4
	10-2	6.10r	3	0		28-2	3.28p	0	4
	10-3	1.37r	2	2		28-3	3.30p	0	4
	11-1	8.58r	1	3		29-1	0.34r	0	0
	11-2	3.82r	0	3		29-2	2.49m	0	1
	11-3	13.65r	2	3		29-3	0.14	0	0
	12-1	5.73r	3	3		30-1	0.53	1	2
	12-2	6.57r	3	3		30-2	14.89c	0	5
						30-3	13.96p	1	4
DEN/125 ng TCDD/kg/day	13-1	8.86r	3	3	Saline/125 ng TCDD/kg/day	31-1	23.64p	1	6
	13-2	21.96r	2	3		31-2	3.91c	0	3
	14-1	7.25r	3	2		31-3	4.41p	0	5
	14-2	21.35r	3	4		32-1	1.22	0	4
	14-3	5.91r	3	2		32-2	1.07p	0	3
	17-1	10.92r	3	2		32-3	9.20r	0	4
	17-2	5.99r	3	2		33-1	1.46p	0	2
	18-1	27.97r	3	2		33-2	1.67	0	2
	18-2	19.21r	3	4		33-3	17.26c	1	2

[a]Distribution of labeled nuclei: r, random; c, centrilobular; p, periportal; m, midlobular. For some low labeling indices, it was not possible to determine a clear distribution pattern of labeled nuclei.
[b]Altered hepatic focus response on hematoxylin & eosin stained sections: 0 = no foci; 1 = a few foci; 2 = moderate number of foci; 3 = many foci.
[c]Toxicity : Severity grades range from 0 = no toxicity to 6 = most severe toxicity observed. Toxicity characterized by cytoplasmic vacuolization, fatty change, bile duct hyperplasia, and/or pigment in Kupffer cells.

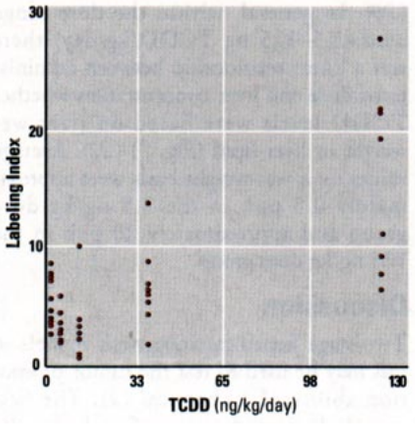


Figure 3. Effect of different doses of TCDD on the bromodeoxyuridine S-phase labeling index in normal hepatocytes.

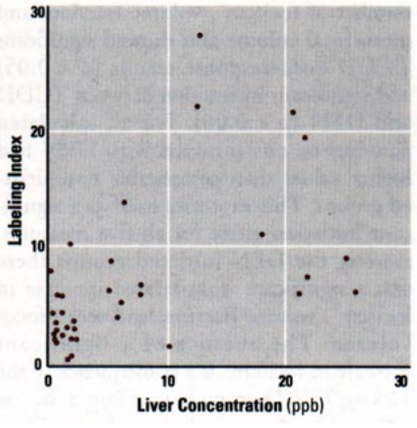


Figure 4. Effect of liver concentrations of TCDD on the bromodeoxyuridine S-phase labeling index in normal hepatocytes.

AHF (*32*). In rats, enzyme histochemical staining for the placental form of glutathione S-transferase is considered a reliable marker for preneoplastic lesions of hepatocytes (*33*). Enhancement of AHF in such initiation-promotion models could be considered a reflection of tumor promotion (*34,35*), as hepatic tumor promoters are known to accelerate the appearance of AHF (*1*) and are able to increase the number and/or size of AHF at a given time interval (*34,36*).

As has been previously demonstrated (*20*), initiation with a necrogenic dose of DEN followed by treatment with 125 ng TCDD/kg body weight/day resulted in development of $PGST^+$ foci. Further confirmation that TCDD acted as a promoter

of putative preneoplastic foci was evident from the significant dose-related increase in PGST⁺ AHF in the TCDD-treated rats as well as increased size and number of AHF noted in H&E-stained sections. Similar to previous results, few PGST⁺ or H&E-stained AHF were present in the TCDD-only treated groups. The increases in number of AHF in initiated rats and in noninitiated rats (less the high dose group) combined with the strong interaction term between TCDD and DEN suggest a complex effect of TCDD.

Previous studies (*22,37,38*) examined dose–response relationships for TCDD-mediated induction of cytochrome P450 isozymes (CYP1A1 and CYP1A2) within the framework of the same liver tumor promotion model described in the present paper. The conclusion, based on both experimental data and biologically based mathematical models for TCDD's effects on gene expression, was that there appears to be a linear relationship between administered dose, liver TCDD concentrations, and cytochrome P450 induction, even in the low dose region used in this study. Therefore, growth and number of preneoplastic lesions appears to occur at a higher dose of TCDD than CYP1A1 or CYP1A2 induction and/or the methods for quantifying putative preneoplastic lesions are inherently less sensitive than are the methods used to quantify cytochrome P450 isozymes. CYP1A1 and 1A2 induction represents a much simpler response, whereas AHF induction takes significantly longer and reflects a more complicated process. This complexity of response for AHF could account for the discrepancy in dose responsiveness when compared to CYP1A1 and 1A2.

There was evidence of cytotoxicity in TCDD-treated groups as supported by histopathological changes, increase in relative liver weight, and alterations in serum clinical chemistry. The morphological evidence of hepatotoxicity was present in all TCDD-treated groups and was more marked in the higher dose groups. In general, hepatocytes were enlarged and vacuolated, without an obvious lobular pattern in the distribution of these changes. The morphological findings are supported by the dose-related increases in relative liver weight and by dose-related alterations in clinical chemistry measurements. Changes in serum cholesterol, AP, and 5′-Nuc are generally reflective of cholestatic changes, whereas changes in SDH and ALT are generally reflective of perturbations in hepatocyte membrane integrity and leakage of these enzymes into the blood. The increases in serum glucose in the noninitiated groups treated with TCDD may reflect perturbation in glucose mobilization in the

Table 4. Proliferating cell nuclear antigen S-phase labeling index (PCNA LI) and growth fraction in altered hepatic foci

Treatment[a]	ID[b]	Mean focus diameter (mm)	PCNA LI (%)	PCNA growth fraction (%)
0 ng TCDD/kg/day	1-1a	0.50	10.71	19.64
	1-1b	0.45	1.47	5.88
	1-2a	0.69	2.42	9.68
	1-2b	0.43	12.70	42.86
	1-4a	0.75	21.09	31.25
	1-4b	0.75	30.43	38.04
	3-1	0.48	18.92	29.05
	3-2a	0.33	2.67	9.33
	3-2b	0.50	8.27	10.53
	3-3a	0.28	54.05	83.78
	3-3b	0.85	27.54	53.29
	3-3c	1.25	36.23	62.32
	Mean	**0.61**	**18.88**	**32.97**
	SE	**0.08**	**4.61**	**7.03**
3.5 ng TCDD/kg/day	4-1a	0.75	26.14	50.57
	4-1b	0.50	13.10	29.17
	5-1	0.63	26.53	52.38
	5-3a	0.40	11.58	22.96
	5-3b	0.50	8.22	11.64
	5-3c	0.85	23.63	47.26
	6-2a	0.50	3.38	6.76
	6-2b	0.55	16.13	24.73
	6-3a	0.35	10.91	19.09
	6-3b	0.35	21.88	27.08
	Mean	**0.54**	**16.15**	**29.16**
	SE	**0.05**	**2.54**	**5.10**
10.7 ng TCDD/kg/day	7-1	0.30	8.75	16.25
	7-3a	0.90	9.80	26.14
	7-3b	0.50	21.69	45.78
	7-3c	0.40	7.29	25.00
	8-2	0.50	39.88	53.18
	8-3a	0.45	18.87	39.62
	8-3b	0.35	23.36	39.42
	9-2a	0.45	1.72	13.79
	9-2b	0.40	0.00	7.69
	9-2c	0.65	3.14	36.48
	9-2d	0.30	0.00	11.76
	9-3	0.75	8.09	15.03
	Mean	**0.50**	**11.88**	**27.51**
	SE	**0.05**	**3.45**	**4.34**
35.7 ng TCDD/kg/day	10-2a	0.53	11.92	23.84
	10-2b	0.90	2.99	37.72
	10-2c	0.35	4.17	50.00
	10-3a	0.58	0.00	3.07
	10-3b	0.48	2.50	6.67
	10-3c	0.48	14.29	17.86
	11-1	0.60	16.78	26.57
	11-3	0.38	4.84	12.90
	12-1a	0.53	21.83	40.85
	12-1b	0.65	7.58	11.36
	12-1c	0.65	2.33	6.20
	2-1d	0.40	42.11	74.74
	Mean	**0.54**	**10.95**	**25.98**
	SE	**0.04**	**3.44**	**6.19**
125 ng TCDD/kg/day	13-1a	0.48	39.00	63.00
	13-1b	0.65	7.50	62.50
	14-1	0.55	44.68	71.49
	14-3a	0.70	4.39	8.77
	14-3b	0.45	3.16	24.21
	17-1	1.00	5.56	45.24
	17-2a	0.78	2.56	6.41
	17-2b	0.35	3.76	6.02
	18-1a	1.05	42.15	50.41
	18-1b	0.70	35.85	44.34
	18-2	0.95	10.53	30.70
	Mean	**0.70**	**18.10**	**37.55**
	SE	**0.07**	**5.41**	**7.21**

[a]All rats initiated with diethylnitrosamine followed by biweekly corn oil or 2,3,7,8- tetrachlorodibenzo-*p*-dioxin treatment.
[b]ID numbers reflect cage number, rat number; a,b,c,d indicate focus identification.

Table 5. Stereologic parameters for placental glutathione S-transferase-positive altered hepatic foci in female Sprague-Dawley rats

Group	Foci/cm^2[a]	Foci/cm^3[b]	Volume fraction (%)[a]	Mean volume ($\mu m^3 \times 10^3$)[a]	Foci/liver[b]
DEN/corn oil	10.9 ± 7.0[c]	442.2 ± 284.4	0.57 ± 0.44[c]	13 ± 6[c]	5748 ± 3923
DEN/TCDD (3.5 ng/kg)	18.4 ± 10.1	759.2 ± 482.0	0.85 ± 0.40	15 ± 12	10552 ± 7941
DEN/TCDD (10.7 ng/kg)	20.4 ± 18.0	791.7 ± 597.2	1.00 ± 1.16	11 ± 4	11482 ± 8879
DEN/TCDD (35.7 ng/kg)	15.6 ± 7.7	530.4 ± 294.8	0.93 ± 0.56	18 ± 7	7157 ± 3952
DEN/TCDD (125 ng/kg)	26.5 ± 15.7*	751.7 ± 428.4	2.23 ± 1.47*	30 ± 8*	11989 ± 6798*
Saline/corn oil	0.4 ± 0.5[d]	22.3 ± 29.0[d]	0.01 ± 0.01	3 ± 3	327 ± 418[d]
Saline/TCDD (3.5 ng/kg)	0.7 ± 1.8	35.9 ± 89.8	0.02 ± 0.05	6 ± 1	457 ± 1122
Saline/TCDD (10.7 ng/kg)	0.6 ± 0.8	34.7 ± 49.8	0.02 ± 0.03	5 ± 3	447 ± 614
Saline/TCDD (35.7 ng/kg)	1.8 ± 2.7	102.8 ± 131.1	0.06 ± 0.11	5 ± 6	1533 ± 1794
Saline/TCDD (125 ng/kg)	1.1 ± 1.6	51.9 ± 69.5	0.04 ± 0.07	5 ± 3	693 ± 921

DEN, diethylnitrosamine; TCDD, 2,3,7,8-tetrachlorodibenzo-*p*-dioxin.
[a]Statistically significant effects for TCDD and initiation with significant interaction between TCDD and DEN ($p < 0.05$) in analysis of variance.
[b]Statistically significant effect of DEN ($p < 0.05$), no significant effect of TCDD in analysis of variance.
[c]$p < 0.05$ for trend test using a general linear model.
[d]Trend is statistically significant when high dose is excluded ($p < 0.05$).
*Statistically significant versus control as measured by two-tailed, unpaired Student's-*t* test, $p < 0.05$.

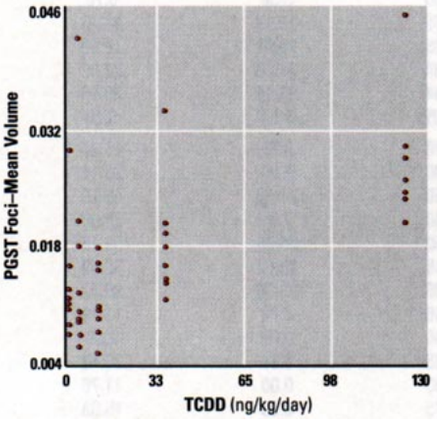


Figure 5. Effect of different doses of TCDD on size (μm^3) of glutathione S-transferase-positive altered hepatic foci in rats initiated with diethylnitrosamine.

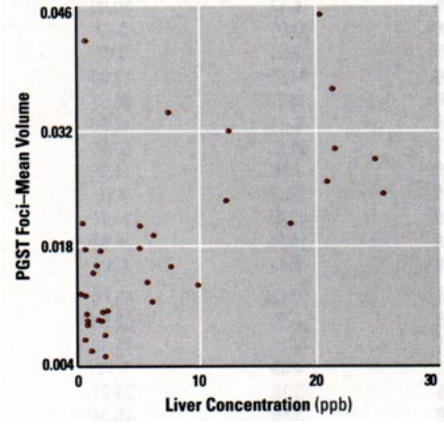


Figure 6. Effect of liver concentrations of TCDD on the size (μm^3) of glutathione S-transferase-positive altered hepatic foci in rats initiated with diethylnitrosamine.

liver, although it is not apparent why similar changes were not detected in the initiated rats treated with TCDD. It is unclear what role DEN is playing in the modification of AP, triglycerides, and SDH. The

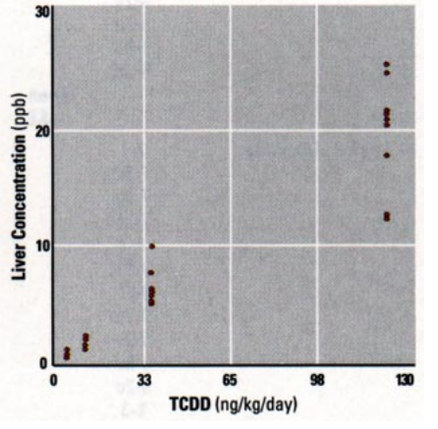


Figure 7. Relationship between administered dose and liver concentration of TCDD in rats initiated with diethylnitrosamine.

evidence of cytotoxicity was also generally supported by the dose-related increase in the rate of replicative DNA synthesis in non-AHF areas of the liver, although there was not a high correlation between LI and toxicity for individual rats. The increased BrdU S-phase LI in DEN-initiated rats versus noninitiated rats may be a consequence of DEN treatment selecting a population of hepatocytes with higher replicative DNA synthesis than those typically present in noninitiated rats.

In the present study, measures of hepatocellular proliferative activity within putative preneoplastic AHF were estimated in DEN-initiated rats by immunohistochemical staining of PCNA. This permitted an estimate of both S-phase hepatocytes within AHF and also the proportion of focus cells active in the cell cycle (growth fraction). Neither of these parameters was significantly influenced by TCDD treatment, suggesting that TCDD does not markedly alter baseline proliferative rates within AHF. Because TCDD does provide a selective growth advantage for AHF as evidenced by increased size of PGST$^+$ AHF (Table 5), it may be that this effect is a result of a decreased rate of cell death within AHF. Alternatively, the ratio of S-phase cells to growth fraction within AHF may be greater than that in non-AHF hepatocytes (not measured in the present study), imparting a selective growth advantage to AHF versus the surrounding hepatic parenchyma. The wide variability in individual AHF proliferative activity within any given experimental group as well as in a given rat suggests that the growth rate of AHF is neither uniform nor synchronized and that AHF may independently respond to positive or negative growth stimuli.

There has been considerable discussion on the advisability of using dose–response relationships for enzyme induction as a surrogate for TCDD's effects on more coordinated biological responses such as cell proliferation or growth of putative preneoplastic lesions. Our data provide evidence that CYP1A1 and CYP1A2 induction may not be a reliable surrogate. First, chronic TCDD doses of 3.5 and 10.5 ng/kg/day produce pronounced increases in CYP1A1 and CYP1A2 protein concentrations and associated enzyme activity (*22*), whereas data reported here indicate less steep responses for replicative DNA synthesis rates in normal hepatocytes. Second, immunolocalization of hepatic CYP1A1 and CYP1A2 induction revealed that induction occurs primarily in the centrilobular region (*22*), whereas our data reveal that cell proliferation in response to TCDD occurred randomly or, in some noninitiated rats, preferentially in the periportal region. Consistent with our evidence on the disassociation between toxic responses and P450 enzyme induction is a recent report by Clark et al. (*24*) which showed that dexamethasone and tumor necrosis factor α blocked the lethal effects of TCDD without effect on the magnitude of P450 enzyme induction.

There was considerable heterogeneity in the cell proliferative responses as well as in AHF development among individual rats within each TCDD-treated group. For example, in the 125 ng TCDD/kg/day DEN-initiated group, approximately half of the rats exhibited markedly elevated LI (18–30%), whereas the other half were only slightly higher than controls (5–10%; Fig. 3). This difference was not correlated with differences in hepatic TCDD concentration, nor was it correlated with the magnitude of CYP1A1 or CYP1A2 induction or histologic evidence of cytotoxicity. Similarly, it is interesting to note that in the chronic bioassay for TCDD carcinogenicity in female Sprague-Dawley rats, approxi-

mately half (40%) the rats at a dose of 100 ng TCDD/kg/day developed hepatocellular neoplasms (*7*). Any differences in AHF response variability in the present study versus a similar previous initiation-promotion study (*12,13*) may be a reflection of differences in the initiation protocols. Although the mechanisms responsible for interindividual differences in TCDD-mediated increases in replicative DNA synthesis rates and AHF formation are not clear, there are some hints that signal transduction events associated with growth factor pathways may be playing a role. For example, TCDD does not induce cell proliferation nor modulate epidermal growth factor receptor actions in ovariectomized rats, whereas significant changes in these parameters are evident in intact female rats (*20, 24*). These findings suggest that TCDD and estrogens can act together to generate a mitogenic signal, a hypothesis that is supported by a recent study which demonstrated that TCDD and estrogens are co-mitogens in rat hepatocyte cultures (*39*).

Data presented in this report attempt to clarify the shape of the dose–response curve for TCDD-mediated increases in cell proliferation and $PGST^+$ foci of cellular alteration within the framework of a rat liver tumor promotion model. Previous studies have demonstrated that TCDD is a potent promoter in two-stage models for liver (*40*) and skin (*41*) cancer and that chronic TCDD exposure enhances cell proliferation rates in hepatocytes of female rats (*20*). While an increase in cell proliferation as estimated by LI was noted in the DEN-initiated rats promoted with 125 ng of TCDD/kg/day, there was a decrease in LI in the 3.5 ng of TCDD/kg/day promoted group, suggesting inhibition of cell proliferation at this low dose. Inhibition of AHF formation at low doses of TCDD has been previous noted (*12*) and has also been reported for phenobarbital promotion in the rat (*42*). This is the first suggestion that a similar phenomenon may be occurring with respect to cell proliferation.

The dose range used in our studies is consistent with the doses given in chronic bioassays for carcinogenicity of TCDD (approximately 1–100 ng/kg/day) (*7,8*). Previous studies in our laboratory demonstrated that few liver tumors develop after 30 weeks of TCDD promotion (100 ng/kg/day) of DEN-initiated rats (*22*). However, after 60 weeks, approximately 50% of the female rats eventually developed tumors (both hepatocellular adenomas and carcinomas) (*24*). Data presented here are consistent with a previous study (*7*) which showed that exposure of rats to 100 ng/kg/day for 30 weeks produced increases in cell proliferation rates and enzyme-altered foci. We extended that finding by evaluating cell proliferation rates and foci of cellular alteration following lower chronic dosing with TCDD. Results indicate that increases in $PGST^+$ foci (mean volume and the proportion of liver occupied by foci) and the LI were statistically significant only at a dose greater than 100 ng/kg/day. Likewise, Pitot et al. (*12*) have shown that significant increases in the volume of liver occupied by enzyme-altered foci were not detected at doses less than 100 ng TCDD/kg/day compared to controls.

Taken together, these and our previous studies (*22,24*) demonstrate that dose-response relationships for TCDD's effects on cell proliferation and growth of AHF are different from those for effects on P450 gene expression. This finding is consistent with the conclusion that different biological or biochemical responses requiring the same receptor (Ah receptor) and the same ligand (TCDD or its structural analogs) may exhibit different dose–response relationships. Thus, the shape of the dose–response curve for receptor-mediated carcinogens may not be predicted solely on the basis that a response is receptor mediated.

References

1. Goldsworthy TL, Hanigan MH, Pitot HC. Models of hepatocarcinogenesis in the rat—contrasts and comparisons. CRC Crit Rev Toxicol 17:61–89(1986).
2. Leonard TB, Dent JG, Graichen ME, Lyght O, Popp JA. Comparison of hepatic carcinogen initiation-promotion systems. Carcinogenesis 7:1797–1803(1982).
3. Grisham JW, Kaufmann WK, Kaufman DG. The cell cycle and chemical carcinogenesis. Surv Synth Path Res 1:49–66(1983).
4. Cohen SM, Ellwein LB. Cell proliferation in carcinogenesis. Science 249:1007–1011 (1990).
5. Swenberg JA, Maronpot RR. Chemically induced cell proliferation as a criterion in selecting doses for long-term bioassays. In: Chemically induced cell proliferation: implications for risk assessment. (Butterworth BE, Slaga TJ, Farland W, McClain M, eds). New York:Wiley-Liss, Inc., 1991;245–251.
6. Butterworth BE, Popp JA, Connolly RB, Goldsworthy TL . Chemically induced cell proliferation in carcinogenesis. In: Mechanisms of carcinogenesis in risk assessment (Vanio H, Magee PN, McGregor DB, McMichael AJ, eds). Lyon:International Agency for Research on Cancer, 1992;279–305.
7. Kociba RJ, Keyes DG, Beyer JE, Carreon RM, Wade CE, Dittenber DA, Kalnins RP, Frauson LE, Park CN, Barnard SD, Hummel RA, Humiston CG. Results of a two-year chronic toxicity and oncogenicity study of 2,3,7,8-tetrachlorodibenzo-p-dioxin in rats. Toxicol Appl Pharmacol 46:279–303(1978).
8. NTP. Bioassay of 2,3,7,8-tetrachlorodibenzo-p-dioxin for possible carcinogenicity (gavage study). Technical Report Series no. 102. Research Triangle Park, NC:National Toxicology Program, 1982.
9. Poland A, Glover E. An estimate of the maximum in vivo covalent binding of 2,3,7,8-tetrachlorodibenzo-p-dioxin to rat liver protein, ribosomal RNA and DNA. Cancer Res 39: 3341–3344(1979).
10. Wassom JS, Huff JE, Lopriano NA. A review of the genetic toxicology of chlorinated dibenzo-p-dioxins. Mutat Res 47:141–160(1977).
11. Kociba R . Evaluation of the carcinogenic and mutagenic potential of 2,3,7,8-TCDD and other chlorinated dioxins. In: Banbury Report 18 (Campbell HA, Poland A, eds). Cold Spring Harbor, NY:Cold Spring Harbor Laboratory, 1984;73–84.
12. Pitot HC, Goldsworthy TL, Moran S, Kennan W, Glauert HP, Maronpot RR, Campbell HA. A method to quantitate the relative initiating and promoting potencies of hepatocarcinogenic agents in their dose-response relationships to altered hepatic foci. Carcinogenesis 8:1491–1499(1987).
13. Pitot HC, Goldsworthy TL, Campbell HA, Poland A. Quantitative evaluation of the promotion by 2,3,7,8-tetrachlorodibenzo-p-dioxin of hepatocarcinogenesis from diethylnitrosamine. Cancer Res 40:3616–3620(1980).
14. Graham MJ, Lucier GW, Linko P, Maronpot RR, Goldstein JA. Increases in cytochrome P-450 mediated 17-b-estradiol 2-hydroxylase activity in rat liver microsomes after both acute administration and subchronic administration of 2,3,7,8-tetrachlorodibenzo-p-dioxin in a two-stage hepatocarcinogenesis model. Carcinogenesis 9:1935–1941(1988).
15. Huff JE, Salmon AG, Hooper NK, Zeise L. Long-term carcinogenesis studies on 2,3,7,8-tetrachlorodibenzo-p-dioxin in the rat. Cell Biol Toxicol 7:67–94(1991).
16. Madhukar BV, Brewster DW, Matsumura F. Effects of in vivo administered 2,3,7,8-tetrachlorodibenzo-p-dioxin on receptor binding of epidermal growth factor in the hepatic plasma membrane of rat, guinea pig, mouse, and hamster. Proc Natl Acad Sci USA 81: 7407–7411 (1984).
17. Hudson LG, Toscano WA Jr., Greenlee WF. Regulation of epidermal growth factor binding in human keratinocyte cell line by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Appl Pharmacol 77:251–259(1985).
18. Romkes M, Piskorska-Pliszczyzynski J, Safe S. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on hepatic and uterine estrogen receptor levels in rats. Toxicol Appl Pharmacol 87:306–314 (1987).
19. Umbreit TH, Gallo MA. Physiological implications of estrogen receptor modulation by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicol Lett 42:5–14(1988).
20. Lucier GW, Tritscher A, Goldsworthy T, Foley J, Clark G, Goldstein J, Maronpot R. Ovarian hormones enhance 2,3,7,8-tetrachlorodibenzo-p-dioxin-mediated increases in cell proliferation and preneoplastic foci in a two-stage model for rat hepatocarcinogenesis. Cancer Res 51: 1391–1397(1991).
21. Abraham K, Krowke R, Neubert D. Pharmacokinetics and biological activity of 2,3,7,8-tetrachlorodibenzo-p-dioxin. Arch Toxicol 62:359–368(1988).
22. Tritscher AM, Goldstein JA, Portier CJ, McCoy Z, Clark GC, Lucier GW. Dose-response relationships for chronic exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin in a rat tumor promotion model: quantification and

immunolocalization of CYP1A1 and CYP1A2 in the liver. Cancer Res 52:3436–3442 (1992).
23. Lucier GW, Rumbaugh RC, McCoy Z, Hass R, Harvan D, Albro P. Ingestion of soil contaminated with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) alters hepatic enzyme activity in rats. Fundam Appl Toxicol 6:364–371 (1986).
24. Clark G, Tritscher A, Maronpot R, Foley J, Lucier G . Tumor promotion by TCDD in female rats. In: Biological basis for risk assessment of dioxins and related compounds (Gallo MA, Scheuplein RJ, van der Heijden CA, eds), Banbury Report 35. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory Press, 1991; 389–404.
25. Maronpot RR, Montgomery CA Jr., Boorman GA, McConnell EE. National Toxicology Program nomenclature for hepatoproliferative lesions of rats. Toxicol Pathol 14:263–273 (1986).
26. Ito N, Tatematsu M, Hasegawa R, Tsuda H. Medium-term bioassay system for detection of carcinogens and modifiers of hepatocarcinogenesis utilizing the GST-P positive liver cell focus as an endpoint marker. Toxicol Pathol 17: 630–641(1989).
27. Campbell HA, Pitot HC, Potter VR, Laishes BA. Application of quantitative stereology to the evaluation of enzyme-altered foci in rat liver. Cancer Res 42:465–472(1982).
28. Pugh TD, King JH, Koen H, Nychka D, Chover J, Wahba G, He Y, and Goldfarb S. Reliable stereological method for estimating the number of microscopic hepatocellular foci from their transections. Cancer Res 43: 1261–1268(1983).
29. Goldsworthy TL, Morgan KT, Popp JA, Butterworth BE . Guidelines for measuring chemically-induced cell proliferation in specific rodent target organs. In: Chemically induced cell proliferation: implications for risk assessment (Butterworth BE, Slaga TJ, eds). New York:Wiley Liss, 1991;253–284.
30. Foley JF, Dietrich DR, Swenberg JA, Maronpot RR. Detection and evaluation of proliferating cell nuclear antigen (PCNA) in rat tissue by an improved immunohistochemcal procedure. J Histotechnol 14:237-241 (1991).
31. Foley JF, Tuck PD, Ton TT, Frost M, Kari F, Anderson MW, Maronpot RR. Inhalation exposure to a hepato-carcinogenic concentration of methylene chloride does not induce sustained replicative DNA synthesis in hepatocytes of female B6C3F1 mice. Carcinogenesis 14: 811–818(1993).
32. Schulte-Hermann R. Tumor promotion in the liver. Arch Toxicol 57:147–158(1985).
33. Sato K. Glutathione S-transferase and hepatocarcinogenesis. Jpn J Cancer Res 79:556–572(1988).
34. Goldsworthy TL, Pitot HC. The quantitative analysis and stability of histochemical markers of altered hepatic foci in rat liver following initiation by diethylnitrosamine administration and promotion with phenobarbital. Carcinogenesis 6:1261–1269(1985).
35. Williams GM. The significance of chemically-induced hepatocellular altered foci in rat liver and application to carcinogen detection. Toxicol Pathol 17:663–674(1989).
36. Xu Y-H, Campbell HA, Satller GL, Hendrich S, Maronpot R, Sato K, Pitot HC. Quantitative stereologic analysis of the effects of age and sex on multistage hepatocarcinogenesis in the rat by use of four cytochemical markers. Cancer Res 50:472–479(1990).
37. Portier C, Tritscher A, Kohn M, Sewall C, Clark G, Edler L, Hoel D, Lucier G. Ligand/ receptor binding of 2,3,7,8-TCDD: Implications for risk assessment. Fundam Appl Toxicol 20:48–56 (1993).
38. Kohn MC, Lucier GW, Clark G, Sewall G, Tritscher AM, Portier CJ. A mechanistic model of effects of dioxin on gene expression in the rat liver. Toxicol Appl Pharmacol 120:138–154 (1993).
39. Schrenk D, Karger A, Lipp H-P, Bock KW. 2,3,7,8-Tetrachlorodibenzo-p-dioxin and ethinylestradiol as co-mitogens in cultured rat hepatocytes. Carcinogenesis 13:453–456 (1992).
40. Dragan YP, Rizvi T, Xu Y-H, Hully JR, Bawa N, Campbell HA, Maronpor RR, Pitot HC. An initiation-promotion assay in rat liver as a potential complement to the 2-year carcinogenesis bioassay. Fundam Appl Toxicol 16: 525–547 (1991).
41. Poland A, Palen D, Glover E. Tumor promotion by TCDD in skin of HRS/J mice. Nature 300:271–273(1982).
42. Maekawa A, Onodera H, Ogasawara H, Matsushima Y, Mitsumori K, Hayashi Y. Threshold dose dependence in phenobarbital promotion of rat hepatocarcinogenesis initiated by diethylnitrosamine. Carcinogenesis 13: 501–503(1992).